

Molecular and Biochemical Characterization of SHV-56, a Novel Inhibitor-Resistant β -Lactamase from *Klebsiella pneumoniae*[▽]

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A clinical strain of *Klebsiella pneumoniae* was found to possess the chromosomal gene *bla*_{SHV-56} encoding a new inhibitor-resistant β -lactamase with a pI of 7.6. SHV-56 is derived from SHV-11 by the single substitution K234R. This mutation therefore evidences a new critical site for inhibitor resistance among SHV enzymes.

Klebsiella pneumoniae is responsible for a wide range of community- and hospital-acquired opportunistic infections, such as urinary tract infections, pneumonia, or septicemia (13). This species is intrinsically susceptible to all antibiotics active against gram-negative bacilli, except to amino- and carboxypenicillins. This low-level resistance is due to the production of a chromosomally mediated broad-spectrum β -lactamase (SHV-1, mainly, or the LEN or OKP type) that is readily inhibited by β -lactamase inhibitors. Some isolates exhibit a high level of resistance to aminopenicillins and reduced susceptibility to amoxicillin-clavulanate and narrow-spectrum cephalosporins due to the acquisition of plasmid-mediated broad-spectrum β -lactamase or overproduction of the chromosomal enzyme (9). Inhibitor-resistant (IR) TEM-type enzymes conferring amoxicillin-clavulanate resistance and cephalothin susceptibility (2, 3, 8, 10) and expanded-spectrum β -lactamases conferring expanded-spectrum cephalosporin resistance (5) have been described in *K. pneumoniae* and other *Enterobacteriaceae*. SHV mutant enzymes are mainly expanded-spectrum β -lactamases (21); however, rare IR-SHV-type β -lactamases (SHV-10 and SHV-49) have been identified (6, 16). We report here SHV-56, the third variant of the SHV family to exhibit resistance to inhibitors, from a *K. pneumoniae* clinical strain.

K. pneumoniae Kp2792 was isolated from the urine of a 68-year-old obese woman hospitalized in 2003 in the infectious diseases ward at the Saint-André hospital, Bordeaux, France. This patient, admitted for leg erysipelas, was first treated by pristinamycin alone. After a febrile episode, amoxicillin-clavulanate was added at 6 g/day intravenously for one and one-half months. By the disk diffusion method, strain Kp2792 exhibited an IR phenotype without coreistances. The MICs, determined by the agar dilution method (<http://www.sfm.asso.fr>), confirmed that Kp2792 was highly resistant to the penicillins tested (MICs of 512 to >4,096 μ g/ml) and susceptible to cephalothin (MIC of 8 μ g/ml) (Table 1). The MICs of penicillins were reduced only slightly by clavulanate and more efficiently by

sulbactam, while tazobactam totally restored the piperacillin susceptibility.

Analysis of the β -lactamase content by isoelectric focusing (11) showed the presence of a single β -lactamase cofocusing with SHV-1 (pI 7.6). The transfer of β -lactamase resistance by conjugation to a nalidixic acid-resistant mutant of *Escherichia coli* K12 by the filter mating technique failed to yield any transconjugant. Plasmid DNA analysis of strain Kp2792 by using a JET STAR plasmid DNA midi kit (Genomed, Löhne, Germany) did not reveal any plasmid. The selection of transformants on agar plates containing ampicillin (100 μ g/ml) after electroporation of putative plasmid DNA extract into *E. coli* JM109 or TOP10 remained unsuccessful. Total DNA of Kp2792 was extracted as previously described (7), and PCR amplification using primers specific for genes encoding IR β -lactamases (*bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA-1}, *bla*_{OXA-2}, *bla*_{OXA-10}, and *bla*_{CARB}) were performed under standard PCR conditions (18). The amplification did not yield any products except for the *bla*_{SHV} gene expected for *K. pneumoniae*. Southern blot analysis followed by DNA-DNA hybridization using Kp2792 total and plasmid DNA preparations, either unrestricted or digested by EcoRI and/or BamHI enzyme; a *bla*_{SHV}-specific probe; and a digoxigenin labeling and detection kit (Roche Applied Science, Meylan, France) confirmed the presence of a single chromosomally located *bla*_{SHV} gene (data not shown). Subsequently, Kp2792 whole-cell DNA was totally restricted by EcoRI and ligated into the EcoRI site of the pBK-CMV cloning vector (Stratagene, La Jolla, CA). *E. coli* JM109 bacteria containing recombinant plasmids were selected on agar plates containing ampicillin (100 μ g/ml) and kanamycin (50 μ g/ml). The recombinant plasmid pC1 containing an insert of ca. 9.5 kb was obtained and subsequently subcloned, using BamHI and EcoRI digestion, into the same vector, giving pSC1 with a ca.-3.5-kb insert. The results of the disk diffusion method and MIC determination demonstrated that *E. coli* JM109 containing pSC1 exhibited the same IR phenotype as strain Kp2792 (Table 1), produced a β -lactamase with a pI of 7.6, and harbored a *bla*_{SHV} gene.

The purified (MicrospinTM Sephacryl S-400; Amersham) *bla*_{SHV} amplicons obtained from strain Kp2792 and recombinant plasmid pSC1 were sequenced on both strands by using a

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TABLE 1. MICs of β -lactams for the clinical isolate Kp2792 and for *E. coli* JM109 strains expressing or not expressing SHV-11 or SHV-56 β -lactamase

Strain	Enzyme produced	MIC ($\mu\text{g/ml}$) of: ^a											
		AMP	SAM	AMX	AMC	TIC	TIM	PIP	TZP	IPM	CEF	CTX	FEP
<i>K. pneumoniae</i> Kp2792	SHV-56	>4,096	128	>4,096	4,096	>4,096	1,024	512	4	≤ 0.1	8	≤ 0.1	≤ 0.1
<i>E. coli</i> JM109(pE1)	SHV-11	1,024	128	2,048	2	2,048	2	64	2	≤ 0.1	8	≤ 0.1	≤ 0.1
<i>E. coli</i> JM109(pSC1)	SHV-56	2,048	128	4,096	512	>4,096	256	512	2	≤ 0.1	8	≤ 0.1	≤ 0.1
<i>E. coli</i> JM109(pE1MD)	SHV-56	1,024	64	4,096	256	2,048	128	64	1	≤ 0.1	8	≤ 0.1	≤ 0.1
<i>E. coli</i> JM109	None	4	2	4	2	4	2	2	1	≤ 0.1	4	≤ 0.1	≤ 0.1

^a AMP, ampicillin; SAM, ampicillin-sulbactam (8 $\mu\text{g/ml}$); AMX, amoxicillin; AMC, amoxicillin-clavulanic acid (2 $\mu\text{g/ml}$); TIC, ticarcillin; TIM, ticarcillin-clavulanic acid (2 $\mu\text{g/ml}$); PIP, piperacillin; TZP, piperacillin-tazobactam (4 $\mu\text{g/ml}$); IPM, imipenem; CEF, cephalothin; CTX, cefotaxime; FEP, cefepime.

DYEnamic ET dye terminator kit (Amersham Biosciences, Orsay, France) and an ABI 310 automatic sequencer (Perkin-Elmer, Courtaboeuf, France). The results of sequence analysis revealed in both cases an identical *bla*_{SHV}-type gene encoding a new β -lactamase variant, named SHV-56. The upstream promoter area showed a C at the second position of the -10 region, thus allowing basal SHV expression (17). *bla*_{SHV-56} differed from *bla*_{SHV-1} (4) by seven nucleotide substitutions that lead to two amino acid substitutions, Leu35→Gln and Lys234→Arg, according to the Ambler nomenclature (1). The Leu35→Gln substitution is frequently encountered and characteristic of the SHV-11 enzyme, which is a broad-spectrum β -lactamase inhibited with a low K_i by clavulanate (6, 12). The Lys234→Arg substitution has not been reported so far, and its role was investigated, especially with regard to β -lactamase inhibitor susceptibility. Thus, site-directed mutagenesis was performed using recombinant plasmid pE1 that expresses the SHV-11 β -lactamase (6), primers MD2792F (5'-GGTTTATC GCCGATAGGACCGGAGCTGG-3') and MD2792R (5'-CC AGTCCGGTCCTATCGGCGATAAACC-3'), and a Quick-Change site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) as indicated by the manufacturer. This technique allowed the lysine at position 234 of SHV-11 to be replaced by an arginine, thus leading to SHV-56. The resulting mutant (pE1MD), whose sequence was double-checked, exhibited an IR phenotype (Table 1).

To further characterize the activity of SHV-56 and compare

its hydrolytic properties to those of SHV-11, the enzymes were extracted as described previously (6) from *E. coli* bacteria containing the plasmids pE1 and pE1MD. The extract was loaded successively onto two Q-Sepharose columns with 20 mM diethanolamine (pH 9.2) and 20 mM Bis-Tris (pH 7) buffer, respectively (15). SHV-56 showed a penicillinase activity similar to that of SHV-11, whereas no hydrolysis was detected against expanded-spectrum cephalosporins (Table 2). The 50% inhibitory concentrations (IC_{50}) of clavulanate, sulbactam, and tazobactam were determined by the rate of benzylpenicillin (100 μM) hydrolysis by purified proteins after 3 min of preincubation at 30°C. The IC_{50} were considerably higher for SHV-56 (2.5 μM and 0.75 μM , respectively) than for SHV-11 (0.12 μM and 0.13 μM , respectively), in contrast with the IC_{50} of sulbactam (0.3 μM for SHV-56 versus 2.4 μM for SHV-11), as confirmed by the MIC results. SHV-56 might display a different turnover number for sulbactam than for clavulanate and tazobactam. These data confirmed that SHV-56 was an IR β -lactamase owing to the Lys234→Arg substitution. Interestingly, position 234 is located in the β -strand of SHV-56, very close to the Ser130 residue, and it takes part in the catalytic site. The amino acid Ser130 plays an important role in substrate turnover and catalysis (proton transfer), and mutations at this site have been previously implicated in site-directed-mutagenetic and natural IR enzymes (3, 19, 20, 22). It could be hypothesized that Arg234, possessing a longer side chain than a lysine at the same position, might

TABLE 2. Kinetic parameters of the purified β -lactamase SHV-56^a

Substrate	SHV-56			SHV-11		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Benzylpenicillin	60	40	1,500	40	20	2,000
Ticarcillin	30	450	70	2	20	100
Piperacillin	50	50	1,000	30	40	750
Cephalothin	0.1	20	5	0.5	50	10
Cefoxitin	<0.01	ND	ND	<0.01	ND	ND
Cefuroxime	<0.01	ND	ND	0.05	200	0.25
Ceftriaxone	<0.01	ND	ND	0.01	300	0.35
Cefotaxime	<0.01	ND	ND	<0.01	ND	ND
Ceftazidime	<0.01	ND	ND	<0.01	ND	ND
Cefepime	<0.01	ND	ND	<0.01	ND	ND
Aztreonam	<0.01	ND	ND	<0.01	ND	ND

^a Data are the means of the results of three independent experiments. Standard deviations were within 10% of the means. The specific activity, measured with 100 μM benzylpenicillin as substrate, was 200 U/mg of protein, with a 20-fold purification factor (14). The kinetic measurements were carried out at 30°C in 100 mM sodium phosphate buffer (pH 7.0) (15). The K_m and k_{cat} values were determined by analyzing β -lactam hydrolysis under initial rate conditions using the Edie-Hofstee linearization of the Michaelis-Menten equation. The minimum detectable hydrolysis value was 0.01 s^{-1} . ND, not determinable due to too-low affinity.

change the Ser130 side chain position and thus its interaction with β -lactamase inhibitors. Moreover, regarding the sulbactam data, the Arg244 position might also be speculated to have a role in the inhibitor resistance.

In conclusion, SHV-56 is a new IR β -lactamase belonging to the SHV family that has been identified in a clinical strain of *K. pneumoniae*. The enzyme was a direct derivative of the naturally occurring SHV β -lactamase in strain Kp2792, and it might have been selected in vivo since the patient had received a long course of treatment with amoxicillin-clavulanate. SHV-56 is derived from SHV-11 by the single substitution Lys234 \rightarrow Arg, which is reported here for the first time in an SHV-type enzyme and has never been observed in natural and artificial IR variants of the TEM family.

Nucleotide sequence accession number. The nucleotide sequence of the *bla*_{SHV-56} gene reported in this work is available in the GenBank nucleotide database under the accession number EU586041.

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